Final Report

Effects of Centrifugation on Gonadal and Adrenocortical Steroids in Rats

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Introduction

Many endocrine systems are sensitive to external changes in the environment. Both the pituitary-adrenal and pituitary gonadal systems are affected by stress including centrifugation stress (Oyama and Daligcon, 1967). The objective of this project is to examine further the effect of centrifugation on the pituitary-gonadal and pituitary-adrenocortical systems by measuring the gonadal and adrenal steroids in the plasma and brain following different duration and intensity of centrifugation stress in rats.

Two studies have been completed and the results are presented below. The second study was carried out to describe the developmental changes of brain, plasma and testicular testosterone and dihydrotestosterone in Sprague-Dawley rats so that the effect of centrifugation stress on the pituitary-gonadal system could be better evaluated in future studies.

Experiment I The Effect of Chronic Hyper-G Stress on the Levels of Dihydrotestosterone, Testosterone and Corticosterone in the Hypothalamus, Cerebellum, Cerebral Cortex and Plasma of the Male Rat

Experimental Design Male Sprague-Dawley rats (Simonsen Labs, Gilroy, CA) were placed on a centrifuge at 34 days of age and maintained at 3.14 g for 146 days. An equal number of rats were placed in the centrifuge room at 34 days of age and maintained there as controls. Both the hyper-G and control animals were fasted overnight prior to the morning of sacrifice. The hyper-G and control animals were moved from the centrifuge room to the place of sacrifice. Thirty minutes following removal from the centrifuge room the animals were rapidly killed by decapitation. Blood samples were collected in heparinized tubes and placed on ice. The heads of the animals were wrapped in aluminum foil and placed on ice until the time of brain removal and dissection. The following brain regions were dissected on a glass plate kept on ice: hypothalamus, cere-

bellum and cerebral cortex. Testes from 2 animals of each group were dissected and analyzed for steroid concentration.

Steroid extraction and separation

Total brains or specific brain areas were homogenized in four volumes of 0.32 M sucrose (DeRobertis, Arnaiz, Alberici, Butcher and Southerland, 1967), transferred to 40 ml glass centrifuge tubes and maintained on ice or frozen until the time of steroid extraction. Testicular tissue was dissected free of fatty tissue and weighed. The capsule was removed and the remaining tissue reweighed and homogenized in 0.32 M sucrose as described above. A 10-20 mg equivalent of testicular tissue was transferred into the 40 ml glass centrifuge tubes for steroid extraction. Where possible 200 µl plasma samples were used for extraction and later separation.

Each sample was then extracted with 23 ml methylene chloride by vigorously inverting the extraction tubes 100-140 times. Following centrifugation for 10 minutes at about 2,000 g, the aqueous layer along with the interface pellet were removed. Twenty ml of the methylene chloride layer was passed through 1.0 gram columns of silica gel (100-200 mesh). This quantitatively removes the steroids concerned while allowing much other material to pass through. The silica gel columns were washed with 6.0 ml of 1% methanol in methylene chloride, while the steroids were eluted batchwise with 8 ml of 5% methanol in methylene chloride. This fraction was evaporated under nitrogen and reconstituted for partition chromatography by dissolving in 20 µl ethanol and 100 µl of an aqueous solution containing 0.1% fructose, 0.02% bovine serum albumin and 0.01 N H_2SO_4.

Celite partition columns were prepared by mixing 2.0 g celite 535 with 1.0 ml of the aqueous solution described above. Packing and associated column preparations have been described elsewhere (Butte, Kakihana and Noble, 1972). Prior to chromatography, Freon 113 (1,1,2-trichloro 1,2,2 trifluoro ethane) was

aspirated through the bottom of the column using vacuum. Thirty ml of Freon was added to the top of each column and allowed to drain through overnight. This procedure dramatically reduced the column blanks. A 100 µl aliquot of the reconstituted sample was placed on each column for steroid separation. The following elution scheme was used throughout this study and is based on an ambient room temperature of 22.5-23.0^{0}C; 100% Freon 113 (4.0 ml-discard; 10.0 ml-dihydrotestosterone fraction; 13 ml-testosterone fraction; 13.0 ml estradiol fraction). The eluted steroids were evaporated to dryness under nitrogen. Identical radioimmunoassay assay procedures were used for dihydrotestosterone and testosterone (Auletta, Caldwell and Hamilton, 1974) using the corresponding standards for each.

Steroid recoveries were determined two ways. The first was done using a pooled male brain homogenate. This sample was assayed repeatedly to determine the endogenous steroid levels. The pooled sample was then reassayed following the addition of known amounts of the steroids allowing recoveries to be determined. When this method was compared to a second method in which standard amounts of steroid in BSA were isolated using the normal procedure and then assayed, the following comparative recoveries were obtained: dihydrotestosterone (52.3%, 67.6%), and testosterone (56.4%, 58.4%) respectively. Since the two methods gave equivalent results the second and easier procedure was routinely used. A minimum of three recovery samples and three column blanks were used with each column series.

Chemicals used were reagent grade where possible. The Freon 113 and methylene chloride were redistilled prior to use in chromatography. Radioactivity was determined in a Packard scintillation counter using a toluene-Triton X114-PPO cocktail (Anderson and McClure, 1973).

Data were analyzed using a 2-way analysis of variance procedure for unequal

numbers per cell (Snedecor, 1940). When significant differences were observed, t-tests were then used to define the differences.

Results and Discussion The results of the steroid analysis are presented in Table 1. Two way ANOVA of the CNS dihydrotestosterone levels revealed significant differences between the centrifuged and control groups ($F=20.20$, $DF=1/28$ $p<.0001$) but no interactive effects ($F=1.65$, $DF=2/28$ $p<0.21$). Testosterone values proved to be different between centrifuged and control groups ($F=4.22$, $DF=1/28$, $p<.05$) and among brain areas ($F=15.98$, $DF=2/27$ $p<.0001$). T-test comparisons were done between control and hyper-G brain levels and plasma dihydrotestosterone and plasma testosterone (Table 1). Testicular weights of two animals from each group are given in Table 2.

The results of this experiment are somewhat limited in scope with certain factors needing better control. The relatively high but nearly identical control and hyper-G plasma and brain corticosterone values suggest that the 30 minutes between the removal of the rats from the centrifuge room caused an environmental stress to take place. This acute stress potentially might alter gonadal steroid levels as well.

The significant differences between brain dihydrotestosterone and testosterone levels are quite interesting in that the hyper-G animals are higher than the controls. There is a general trend toward hyperfunction of the pituitary-gonadal system in the chronically hyper-G adapted animals. The significant elevation of plasma testosterone in the centrifuged animals as well as a tendency toward testicular hypertrophy (Table 2) support this. If this were to hold true, sexual behavioral testing would be valuable.

Experiment II Plasma, Testicular and Brain Region Concentrations of Testosterone and Dihydrotestosterone in Developing Rats

Animals

Sprague-Dawley derived male rats were purchased from Simonsen Laboratories, Gilroy, CA and housed in the animal facilities of San Francisco State University a minimum of 6 days before sacrifice. Ten day old animals were born in our laboratories, the offspring of timed pregnancy Sprague-Dawley rats purchased from Simonsen Laboratories shortly after mating. All animals were maintained on Wayne Lab Blox rat chow and water ad libitum at 22°C with a 12-hr light-dark cycle (0600-1800). Animals were sacrificed between 9:00-12:00 hr.

Hormone determinations were carried out in the following age groups: 10, 30, 39, 50 and 68 days. For 10-day old rats blood samples were collected from the heart following ether anesthesia. For all other age groups blood was obtained from the abdominal aorta following ether anesthesia. The entire procedure of blood collection was kept under three minutes to minimize the possibility of steroid alterations due to the stress of obtaining the samples. The brains were perfused by the injection of 30-50 ml of ice cold saline into the heart. The following brain regions were dissected on a glass plate kept on ice: hypothalamus, cerebellum, cerebral cortex and pituitaries. The brain areas and plasma samples were maintained on ice until homogenization and centrifugation. Four 10-day old rat brain area and plasma samples were combined for a single-steroid analysis series. Samples from two rats were combined for a single determination for the remaining age groups. Testes were dissected and trimmed of fat tissues and homogenized as described for the brain tissue.

Subcellular distribution of testosterone, dihydrotestosterone and estradiol in male rat brain

In a separate study brain areas from 10 68-day old male rats were analyzed

for testosterone, dihydrotestosterone and estradiol following the subcellular fractionation procedure of DeRobertis et al. (1967). This study was replicated in another group of 10 comparable rats.

Results and Discussion

Dihydrotestosterone and testosterone levels in the hypothalamus, cerebellum, cerebral cortex and pituitary during the postnatal development of the male rat are presented in Table 3. Two-way analysis of variance of the dihydrotestosterone levels (excluding the pituitary values) indicates significant differences among the brain areas ($F=12.45$, $DF=2/65$ $p<.001$), among ages ($F=44.28$, $DF=4.65$ $p<.0001$) and an interactive effect as well ($F=2.25$, $DF=9/65$ $p<.05$). Similar analysis was done on the brain testosterone levels (again excluding the pituitary values). Significant area differences ($F=9.29$, $DF=2.65$ $p<.0003$), age differences ($F=92.97$, $DF=4/65$ $p<.0001$) and interactive differences ($F=4.23$, $DF=8/65$ $p<.0004$) were also found. A two-way analysis of variance comparison of each brain area paired with a second brain area demonstrated that hypothalamic dihydrotestosterone levels were higher than those of the cerebellum ($F=4.93$, $DF=1/43$ $p<.04$) and cerebral cortex ($F=23.83$, $DF=1/44$ $p<.0001$) while the cerebellum was higher than the cerebral cortex ($F=8.71$, $DF=1/43$ $p<.006$). Although highly significant age differences were found in all comparisons only the hypothalamic-cerebral cortex comparison demonstrated significant interactive effects ($F=3.35$, $DF=4/44$ $p<.02$). A similar paired comparison between brain area testosterone levels demonstrated that hypothalamic levels were higher than cerebellum levels ($F=10.45$, $DF=1/44$ $p<.003$) with significant interaction ($F=5.44$, $DF=4/44$ $p<.002$), but were not higher than cerebral cortex levels ($F=0.86$, $DF=1/43$ $p<.36$) although interaction effects were significant ($F=3.27$, $DF=4/43$ $p<.02$). T test comparisons (shown in Table 3) indicated that significant increases in steroid levels occurred between days 10 and 30 as well as 39 and 50. Hypotha-

lamic testosterone also increased between days 50 and 68. Dihydrotestosterone was significantly higher than testosterone in both the hypothalamus and cerebellum on day 30 while testosterone was significantly higher than dihydrotestosterone in the cerebral cortex on days 50 and 68. No other differences reached the .05 level of significance.

Plasma and testicular levels of dihydrotestosterone and testosterone are presented in Table 4. Two-way analysis of variance of the plasma steroid levels demonstrated significant age differences ($F=25.53$, $DF=4/42$ $p<.001$), and significant differences between dihydrotestosterone and testosterone levels ($F=79.63$, $DF=1/42$ $p<.001$). Plasma testosterone levels were greater than the corresponding dihydrotestosterone at all ages ($p<.03$). Significant changes in levels occurred between age 10 and 30 days as well as 39 and 50 days. These are indicated in Table 4. Differences existed in the testicular samples between the dihydrotestosterone and testosterone levels ($F=30.60$, $DF=1/34$ $p<.001$), among ages ($F=10.94$, $DF=4/34$ $p<.001$) as well as a strong interaction effect ($F=14.60$, $DF=4/34$ $p<.001$). Testosterone and dihydrotestosterone levels were statistically different ($p<0.02$) at every age group tested except day 50 rats ($p<0.47$). Significant differences in steroid levels between the different adjacent age groups are indicated in Table 4. Only the levels measured on age 30 and 39 day animals failed to be significantly different.

The results of the subcellular analyses of testosterone, dihydrotestosterone and estradiol in the hypothalamus, cerebellum and cerebral cortex are presented in Table 5. Since two separate experiments were conducted, the values from each set are presented under separate column, a and b in Table 5. Replication seems quite good since corresponding values from the two studies are amazingly close.

Although a large percentage of these gonadal hormones are found in the

supernatant fraction of the nerve tissues, a substantial amount is found in myelin fraction as well. In addition it is to be noted that the proportions of the steroids in the nuclear fraction are relatively small in comparison to supernatant fractions, although the primary function of steroids in the nerve tissue is presumed to be in the nucleus. Since no other comparable data are available, it is difficult at this point to relate these subcellular data to functional significance. We feel that perhaps similar analyses in physiologically altered conditions, such as chronic stress-induced hypogonadism or prenatally feminized males, might shed some light on the function of gonadal steroids in controlling the CNS functions.

At the present time the differences in steroid levels between the hyper-G adapted animals and their controls cannot be explained on a behavioral basis. The higher brain levels of dihydrotestosterone and testosterone as well as higher plasma testosterone levels in the hyper-G adapted animals suggest a hyperfunction of pituitary-gonadal axis in these animals. The limited number of testicular samples tends to support this hyperfunction. The question as to whether these differences would cause concomitant sexual differences requires behavioral testing not attempted in the present studies.

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Table 1. The effect of chronic hyper-G stress on dihydrotestosterone, testosterone and corticosterone in the plasma and brain areas of male rats

	Plasma		Hypothalamus		Cerebellum		Cerebral Cortex	
	Control	Hyper-G	Control	Hyper-G	Control	Hyper-G	Control	Hyper-G
Dihydrotestosterone	0.073 ± .015	0.106 ± .016	0.287 ± .060	0.542* ± .068	0.119 ± .023	0.287* ± .051	0.091 ± .014	0.178* ± .027
Testosterone	0.679 ± .138	1.574* ± .208	1.118 ± .216	1.509 ± .333	0.237 ± .056	0.462** ± .073	0.305 ± .041	0.626* ± .066
Corticosterone	30.1 ±2.14	29.4 ±4.23	7.09 ±1.85	6.55 ±1.93	3.87 ±0.76	4.20 ±0.79	4.18 ±0.50	4.83 ±1.10

Dihydrotestosterone and testosterone levels are presented as ng/ml or g

Corticosterone is presented as µg/100 ml or g

All values except hyper-G cerebellum testosterone (n=5) are the means of six rats

*Significantly higher than the control value ($p<.03$)

**Significantly higher than the control value ($p<.05$)

Table 2 Testicular weights and steroids of control (n=2) and hyper-G adapted (n=2) rats

	Control	Centrifuged
	Grams	
Absolute Weight	2.527 (2.672) 2.816	2.980 (2.940) 2.899
	Grams/kg	
Relative Weight	4.595 (5.108) 5.621	7.045 (6.933) 6.821
	Ng/g	
Dihydrotestosterone	2.37 (1.55) 0.72	2.64 (3.13) 3.61
	Ng/g	
Testosterone	60.7 (41.6) 22.4	69.7 (62.7) 55.7

Table 3. Brain area levels of dihydrotestosterone and testosterone in the developing male rat

	Ng/gram tissue				
Dihydrotestosterone	Day 10	Day 30	Day 39	Day 50	Day 68
Hypothalamus	0.143±0.048(5)	0.903±0.200(6)	0.544±0.065(4)	1.428±0.203(6)	1.999±0.178(6)
Cerebellum	0.109±0.023(5)	0.562±0.117(6)	0.576±0.111(4)	1.378±0.183(6)	1.308±0.131(5)
Cerebral Cortex	0.075±0.016(5)	0.418±0.081(6)	0.447±0.053(4)	0.837±0.099(6)	1.029±0.162(6)
Pituitary	0.704(1)	2.11(1)	1.29(1)	1.07(1)	0.860±0.120(4)
Testosterone					
Hypothalamus	0.113±0.037(5)	0.323±0.082(6)	0.421±0.083(4)	1.322±0.206(6)	2.536±0.267(6)
Cerebellum	0.091±0.022(5)	0.244±0.059(6)	0.299±0.057(4)	1.211±0.138(6)	1.372±0.129(6)
Cerebral Cortex	0.102±0.025(5)	0.349±0.074(5)	0.523±0.078(4)	2.128±0.205(6)	2.121±0.221(6)
Pituitary	1.071(1)	0.40(1)	0.52(1)	1.31(1)	2.30 ±0.530(4)

The values are mean ± SEM. The numbers in the parenthesis show the number of determinations

Table 4. Plasma and testicular levels of dihydrotestosterone (DHT) and testosterone (T) in the developing rat (ng/ml or g)

	Plasma		Testis	
Age (days)	DHT	T	DHT	T
10	0.038±0.005(5) p<.001	0.222±0.028(5) p<.08	5.83±1.31(5) p<.006	109.6 ±27.5(5) p<.006
30	0.157±0.020(5)	0.496±0.132(6)	14.8 ±2.02(5)	5.07± 0.78(5)
39	0.158±0.026(4) p<.01	0.775±0.213(4) p<.01	14.6 ±2.07(4) p<.002	7.26± 0.96(4) p<.001
50	0.320±0.040(6)	2.672±0.341(5) p<.05	27.9 ±1.44(4) p<.007	29.9 ± 2.17(4) p<.003
68	0.373±0.049(6)	4.325±0.614(6)	12.0 ±3.63(4)	130.3 ±20.7(4)

Values are mean ± SEM. Numbers in the parentheses show the number of determinations.

T-test comparisons when shown are between adjacent age groups.

Table 5. Subcellular distribution of dihydrotestosterone (DHT), testosterone (T) and estradiol (E_2) in three brain areas of the 68-day old male rat

	HYPOTHALAMUS					CEREBELLUM				CEREBRAL CORTEX			
	DHT		T		E_2	DHT		T		DHT		T	
	a	b	a	b	a	a	b	a	b	a	b	a	b
Supernatant	38.1	56.8	68.9	64.4	13.6	29.0	31.1	46.2	30.2	30.9	36.6	47.5	57.4
Nuclear	9.1	6.3	4.8	2.7	23.1	21.3	25.3	15.1	14.0	8.4	4.5	5.9	2.8
Microsomes	19.1	19.8	11.3	8.6	10.7	13.3	15.6	14.0	9.0	16.6	21.1	14.6	15.0
Myelin	22.7	15.3	13.0	9.6	35.9	18.8	18.1	17.8	18.3	20.8	18.9	23.8	12.9
B	9.6	1.8	1.1	5.7	16.7	11.2	8.0	5.5	19.7	17.5	14.2	7.2	6.2
C	0.7	0.0	0.4	6.3	0.0	4.6	1.1	0.8	1.2	3.2	3.5	0.6	3.0
D	0.5	0.0	0.2	2.3	0.0	0.9	0.6	0.2	4.3	1.1	0.0	0.2	0.0
Mitochondria	0.2	0.0	0.4	0.3	0.0	0.9	0.1	0.3	3.3	1.5	1.3	0.2	2.8

The values are presented as the % of steroid each fraction contains relative to the total amount present